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TITLE

Inbreeding, immune defence and ectoparasite load in different mockingbird populations and species in the Galápagos Islands

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RUNNING TITLE

Inbreeding and immunity of mockingbirds

ABSTRACT

Inbreeding may impair an individual's immune system, render it more susceptible to disease and hence contribute to the extinction risk of small and isolated populations, as often found on islands. So far, surprisingly few studies have assessed the effects of inbreeding on immunocompetence in wild populations. Using 26 microsatellite loci and genetic data from museum specimens and contemporary samples, we calculated short-term and long-term inbreeding in 13 different mockingbird populations covering the range of all 4 species in the Galápagos Islands and compared them with three different measures of innate immunity and ectoparasite load. We found no significant effect of either measure of inbreeding on natural antibody or complement enzyme titres, heterophil-lymphocyte ratio or feather louse abundance. Hence, our results do not support a link between inbreeding and immunocompetence. However, overall statistical power and repeatabilities of antibody and complement enzyme titres were low. Nevertheless, generally, natural antibody titres were high suggesting that the mockingbirds may be equipped with a strong first line of defence, as found in other island species.

KEYWORDS

Genetic diversity, immunocompetence, birds, lice, natural antibodies

INTRODUCTION

Small and isolated populations are not only more susceptible to demographic and environmental stochastic events (Reed 2004), but also suffer from inbreeding and a faster loss of genetic diversity through genetic drift (Allendorf and Luikart 2007). One way in which inbreeding and loss of genetic diversity may increase extinction risks of small populations is by making them more vulnerable to infectious diseases (e.g. McCallum and Dobson 1995, Smith et al. 2009). Inbred individuals and populations have been shown to exhibit a decrease in parasite or pathogen resistance (e.g. Cassinello et al. 2001, Coltman et al. 1999, Puurtinen et al. 2004, Pearman and Garner 2005, Acevedo-Whitehouse et al. 2003, Hedrick et al. 2001, Ross-Gillespie et al. 2007) or lowered immune response (e.g. Reid et al. 2007, Reid et al. 2003), albeit not universally (e.g. Giese and Hedrick 2003, Gerloff et al. 2003, Sandland et al. 2009). Results are therefore still contradictory and more empirical data are needed to evaluate the overall impacts of inbreeding on the ability of populations to cope with disease under natural environmental conditions (Smith et al. 2009, Keller and Waller 2002, Spielman et al. 2004, Hawley et al. 2010).

Small host populations on isolated islands are particularly susceptible to foreign pathogens (McCallum and Dobson 1995), and diseases have caused dramatic population declines and extinctions of avian species on islands (Van Riper et al. 2002, Van Riper et al. 1986). In the Galápagos Islands, the introduction of alien avian diseases has become a major concern leading to the initiation of a number of avian health survey projects (Gottdenker et al. 2005, Soos et al. 2008, Parker et al. 2006, Wikelski et al. 2004), including a disease survey of the endangered Floreana mockingbird (*Mimus trifasciatus*; Deem et al. 2011). *Mimus trifasciatus* is one of four endemic, allopatrically living mockingbird species found in the Galápagos (Harris 1974) and, since its extinction from the main island of Floreana in the late 1800s, its distribution has been restricted to two small satellite islands

(Fig. 1). In a recent study that covered the range of all four Galápagos mockingbird species (Fig. 1) and many different populations, we detected pronounced differences in average inbreeding levels among species and populations (Hoeck et al. 2010b) rendering these isolated populations of different size an ideal natural system to study the effects of genetic diversity on different immune components. Additionally, the availability of genetic data from historical samples over 100 years old allows differentiating between recent inbreeding vs. historically low levels of genetic diversity (Hoeck et al. 2010b). This is of interest because populations that have undergone historical processes of inbreeding may have successfully purged some of their immunity-related genetic load (Crnokrak and Barrett 2002, Ross-Gillespie et al. 2007), resulting in a weaker association between inbreeding and immunity.

In this study, using genetic data from historic and contemporary samples, we determined levels of genetic diversity and inbreeding at the population level and their effects on immunocompetence, i.e. the ability to prevent or control infections by pathogens and parasites (Norris and Evans 2000). We studied 13 populations covering the range of the four mockingbird species in the Galápagos (Fig. 1) and, using two different measures of Fst as estimates of population-level inbreeding, we investigated the relationship between inbreeding and multiple immune parameters. We tested a) the innate immunity by counting different types of white blood cells, b) the innate humoral immune response by assessing natural antibody and complement enzyme activity, and c) determined ectoparasite load to test for a potential relationship between inbreeding and parasite burden.

The avian immune system relies on three major defence mechanisms to resist infection by parasites and disease, namely innate immunity, and humoral and cell-mediated acquired immunity (Cheng and Lamont 1988). All three components have been shown to be under genetic control (Sarker et al. 2000) and could hence be affected by inbreeding. Innate immune defences are constitutive and induced rapidly and therefore most important against first exposures to pathogens and quickly growing infections, resulting in non-specific defence

94 reactions such as inflammatory reaction or phagocytosis. The typical response to infectious
95 or inflammatory diseases in birds is an increase in the total white blood cell (WBC) count.
96 Increased lymphocyte numbers have been detected in infected individuals (e.g. Ricklefs and
97 Sheldon 2007, Apanius et al. 2000) and high ratios of heterophils to lymphocytes (H:L ratios)
98 in blood samples reliably indicate stress (Davis et al. 2008). Therefore, the
99 heterophil:lymphocyte ratio has been suggested to serve as a measurement of infection
100 status and physiological stress in poultry (e.g. Gross and Siegel 1983, Maxwell 1993) and
101 wild birds (Tompkins et al. 2006). We determined relative abundances of heterophils and
102 lymphocytes to test for a link between inbreeding and H:L ratio.

103 Although innate immunity mediated by phagocytic and bactericidal WBCs represents
104 a potent first line of defence against pathogens, natural antibodies (NAbs) provide a
105 supplementary or alternative constitutive defence (e.g. Ochsenbein and Zinkernagel 2000,
106 Zouali 2001). As they are directly encoded in nuclear DNA (Belperron and Bockenstedt 2001)
107 and levels have been shown to respond to selection on other immune components in
108 chickens (Parmentier et al. 2004), genetic differences may influence their titres. NAb levels
109 may hence covary with genetic diversity, as has been shown for other immunologically
110 important proteins in vertebrates such as MHC (Miller and Lambert 2004). Furthermore, NAb
111 response is hypothesized to predict the strength of the adaptive immune response (Kohler et
112 al. 2003), providing a link between the innate and acquired humoral immunity (Lammers et
113 al. 2004, Ochsenbein and Zinkernagel 2000). Together with the complement enzymes, NAbs
114 initiate the complement enzyme cascade which eventually leads to cell lysis (Carroll and
115 Prodeus 1998). In a hemolysis-hemagglutination test, we determined agglutination (which
116 arises from NAbs only) and lysis (reflecting an interaction between NAbs and complement
117 enzymes) to estimate the strength of the constitutive innate immune system (Matson et al.
118 2005).

We also tested whether inbreeding correlates with ectoparasite load by determining the abundance of two different louse lineages in the plumage of the mockingbirds (Stefka et al. 2011). Ectoparasites encounter host immune defences when they feed on blood or living tissue (Marshall 1981) and may therefore stimulate a spectrum of different immune responses that potentially impair their development or even kill them (Wikel and Alarcon-Chaidez 2001). A link between genetic diversity and immunity and/or ectoparasite abundance was detected in some cases (Whiteman et al. 2006, Luong et al. 2007), whereas no such correlation was found in others (Taylor and Jamieson 2007).

We made the following predictions: a) If inbreeding negatively affects immunocompetence, e.g. through deleterious mutations or less variable immune components, more inbred populations should show lower levels of average innate and innate-humoral immunity and carry more ectoparasites (i.e. show higher infestation intensity) relative to outbred populations, and b) populations that experienced relatively recent, fast inbreeding are expected to show lower immunocompetence and defence against ectoparasites than populations with a long history of low genetic diversity.

MATERIALS AND METHODS

Sample collection

We visited the Galápagos Islands from December-March in three consecutive years (2006-2008) and collected samples on 11 different islands and at 13 different locations (Fig. 1 & Table 1). On St. Cruz and San Cristóbal, we obtained samples from two different locations, separated by approx. 10 km and 35 km, respectively. We distinguished between the two locations on the same island as previous genetic analyses detected substantial within-island genetic differentiation (Hoeck et al. 2010b). Four islands (Champion, Gardner-

144 by-Floreana, Española and St. Cruz) were visited in all three years to account for variation
145 between years in the parameters estimated here.
146 Mockingbirds were captured in potter traps and, in some areas, also in mist nets and
147 bled immediately after capture. Blood samples were obtained by a small puncture of the wing
148 vein collecting between 20-140 µl of blood (less than 1% of the body weight of a bird) in
149 heparinized microtubes. The blood was transferred immediately into Eppendorf tubes for
150 centrifugation on site. Small drops of blood were used to produce two blood smears and one
151 drop was transferred onto filter paper treated with 0.5 M EDTA for later DNA extraction.
152 Blood smears were air-dried and fixed and stained with Diff-Quick solution (Medion
153 Diagnostics GmbH, Düdingen, Switzerland). Whole blood was centrifuged in the field and
154 plasma was transferred into a liquid nitrogen container where it was kept frozen until used for
155 the hemolysis-hemagglutination assay in the lab in Switzerland.
156
157 *White blood cell counts*
158 Blood smears were examined under 1000 x magnification with oil immersion and the
159 proportion of different types of white blood cells (WBCs), i.e. heterophils, lymphocytes,
160 eosinophils, basophils and monocytes, was assessed by counting a total of 100 WBCs. All
161 counts were performed by the same person (PEAH). To estimate variation within the same
162 smear (i.e. repeatability), we repeated the count of 100 WBCs for 20 different individuals.
163 Additionally, to estimate variation between two smears taken from the same individual at the
164 same time, we also analysed the second smear of 22 individuals. We only used data for
165 lymphocytes and heterophils as the most numerous WBCs in our analyses.
166 As a higher number of WBCs relative to red blood cells (RBCs) can indicate greater
167 innate immune capacity (Zuk and Johnsen 1998), we also counted the relative abundance of
168 WBCs in a subsample of 10 individuals for each of seven populations sampled in 2007.
169 Relative WBC numbers were determined using a slide scanner (Zeiss Mirax Midi Slide
170 Scanner, Carl Zeiss Inc.) and selecting a field covering approx. 10,000 - 40,000 RBCs. RBCs

were counted automatically with the scanner's software (Pannoramic Viewer 1.14.50), and WBCs in the scanned field were subsequently counted by eye.

Hemolysis-hemagglutination assay

Agglutination and lysis titres were assessed with a hemolysis-hemagglutination assay as described in Matson et al. (2005), with higher scores indicating more effective immune responses. Plasma samples were placed on plates randomly, serially diluted twofold with saline and incubated with rabbit red blood cells (Harlan Laboratories UK Ltd.) for 90 min at 37°C. Because we only obtained small volumes of plasma from most mockingbirds, we omitted the first, undiluted row in the assay, hence working with 25 µl of plasma and a dilution series from 1/2 to 1/4096. A plasma sample from a single chicken individual (Harlan Laboratories UK Ltd.) was used as a control. We determined the dilution step at which either the agglutination or lysis reaction stopped (titre score) and took digital images. Titre scores were confirmed a few days later using the digital images only. All scoring was carried out blindly with respect to the identity of the individual birds and was always performed by the same person (PEAH). Sixteen individuals were tested on two different plates to estimate within-individual repeatability among different assay plates. To account for differences between plates processed at different times, all mockingbird lysis and agglutination scores were corrected for the chicken control sample (that was run at the same time) by subtracting the score of the chicken from the mockingbird scores.

Ectoparasite collection and counting

To quantify ectoparasite load, we dust-ruffled a subset of mockingbirds (Table 1) following the method described in Walther and Clayton (1997) by applying 0.7g of pyrethron powder (non-toxic to birds; 0.3% natural flower-extract pyrethrum and 1% piperonyl butoxid; Vetyl-Chemie GmbH, Germany) to the plumage of the birds (all feather tracts except the head). Dust-ruffling time was kept short to reduce stress for the birds under the hot field

198 conditions. Dusting was performed for 2.5 min, followed by 1 min of incubation and 2.5 min
199 ruffling over a clean plastic tray to extract ectoparasites which were subsequently stored in
200 97% ethanol until counted and identified in the lab. Only adult and nymphal lice were
201 considered for further analyses as they were the largest and clearly most abundant
202 ectoparasites detected (77% of all organisms detected in the plumage). Identification of louse
203 genera was done by Vincent Smith, Natural History Museum, UK. Lice belonged to two
204 different genera, *Brueelia galapagensis* (Ischnocera) and *Myrsidea nesomimi* (Amblycera).
205 Because *Brueelia* belongs to the feather-chewing lice, it is unlikely to have a direct effect on
206 the birds' immune system, whereas *Myrsidea* lice may cause an immune reaction through
207 their tissue- and blood-feeding behaviour (Moller and Rozsa 2005). However, because
208 feather-chewing lice are also known to have negative effects on host fitness (Booth et al.
209 1993, Clayton et al. 1999) and might select on behavioural or physical host defences that
210 may be sensitive to host genetic diversity (Whiteman et al. 2006), we included both *Myrsidea*
211 and *Brueelia* lice in our analyses.
212
213 *DNA extraction and microsatellite analysis*
214 Extraction of DNA from blood on filter paper and amplification at 26 microsatellite loci
215 (MpAAT18, 25, 26, 45, 83 and 96, and Nes01, 03, 04, 05, 06, 07, 08, 10, 11, 12, 13, 14, 15,
216 16, 17, 18, 19, 20, 22 and 23) were performed as described previously (Hoeck et al. 2009).
217 Microsatellite loci were amplified in six independent multiplex reactions (Panel A-D, Hoeck et
218 al. 2009; Panel E with MpAAT18, 25, 45 and 83; Panel F with MpAAT26 and 96 under the
219 same PCR conditions as Panel B & C). Fragment analyses were performed on a 3730 DNA
220 Analyser using Gene-Scan-500 LIZ size standard (ABI) and Genemapper v.4 software (ABI)
221 followed by manual proofreading of genotypes.
222 Because morphology-based sexing is difficult, we performed molecular sexing by
223 amplifying the CHD-W and CHD-Z genes (Griffiths et al. 1998) using redesigned primers
224 (Hoeck et al. 2009).

225

226 *Genetic diversity and inbreeding estimates*

227 Hardy-Weinberg equilibrium for each locus was tested with allele randomizations
228 within samples (1,000 permutations per test) and overall samples (10,000 permutations)
229 using FSTAT 2.9.3.1 package (Goudet 2001) and adjusting for multiple comparisons using
230 Bonferroni corrections. Genotypic equilibrium between all pairs of loci in each population was
231 tested using G-statistics with Bonferroni corrections and 84,500 permutations in FSTAT.
232 Within population genetic diversity was estimated using standard genetic parameters such as
233 number of alleles (NA), allelic richness (AR, standardized to the smallest sample size) and
234 expected heterozygosity (He) as calculated in FSTAT. As NA and AR correlated strongly with
235 He (NA: $r^2 = 0.89$, $p < 0.001$; AR: $r^2 = 0.94$, $p < 0.001$), we only used He as a measure of
236 population genetic diversity in our analyses.

237 Inbreeding is always a relative measure and different estimates depend on their
238 population of reference (Keller and Waller 2002). Hence, they also relate to different time
239 periods in the past during which inbreeding could accumulate (Biebach and Keller 2010). In
240 randomly mating populations, overall inbreeding can be expressed as Wright's Fst (Keller
241 and Waller 2002). Here, we calculated two different measures of Fst to distinguish between
242 two different time scales during which inbreeding accumulated in each population: recent (i.e.
243 short-term) inbreeding and long-term inbreeding. As a measure of recent inbreeding we used
244 the previously published "temporal Fst" Table 1 (Hoeck et al. 2010b). This temporal Fst
245 quantifies the increase in inbreeding within each island/population over the last century by
246 comparing the genetic diversity of museum specimens collected in 1906 to the one found for
247 the contemporary samples (Hoeck et al. 2010b). The historic data set consisted of 302
248 specimens from all 11 islands investigated here (sample size: 11-29 specimens/island) that
249 were genotyped at 16 microsatellite loci. These 16 loci were used to quantify changes in
250 microsatellite allele frequencies since 1906, i.e. temporal differentiation (temporal Fst)
251 between historic and contemporary samples within each island (Hoeck et al. 2010b). To

quantify long-term inbreeding, we calculated population-specific Fst values (Biebach and Keller 2010) using the software 2mod (Ciofi et al. 1999) and selecting the "infer model" mode that we ran with 500,000 iterations. Inbreeding can occur due to both, subdivision in finite populations and non-random mating within subpopulations, and overall inbreeding is given by (1-Fit) = (1 - Fis)(1 - Fst) (Wright 1969, p. 295). Since we did not detect any inbreeding due to non-random mating, i.e. Fis=0 (it was not significantly different from zero in any population, see below), overall inbreeding equals Wright's Fst (i.e. Fit=Fst; Keller and Waller 2002). We call this Fst "population-specific Fst" because it quantifies total inbreeding that accumulated due to drift (as there is no inbreeding due to non-random mating) relative to the last common ancestral population (Biebach and Keller 2010).

Heterozygosity-heterozygosity correlations

The use of microsatellite markers to predict levels of inbreeding has been questioned (e.g. Balloux et al. 2004, DeWoody and DeWoody 2005, Ljungqvist et al. 2010). We therefore estimated the degree to which heterozygosity is correlated across unlinked markers following the method described in Balloux et al. (2004): assuming that microsatellite and genome-wide heterozygosity are correlated, heterozygosity estimated from one set of microsatellites should be positively correlated with heterozygosity from an independent set of microsatellites from the same individual ('heterozygosity–heterozygosity correlations'). We randomly split the 26 loci into two sets of 13 independent loci and performed the analysis, a) for each population separately to estimate heterozygosity-heterozygosity correlations on the individual level within a population, and b) in a separate analysis, including all populations and individuals to estimate correlations at the population level, both times using 1,000 iterations to calculate the distribution of the correlation coefficients between the two sets of loci. We detected high correlations when including all individuals and populations in the analysis (mean correlation coefficient: 0.65 ± SD 0.03), but low or no correlation when doing analyses for each population separately (correlation coefficients ranging from -0.24 to 0.16 with

279 distributions including 0). We therefore did not include individual multilocus heterozygosity in
280 any of our statistical models, but restricted our inbreeding – immunocompetence analyses to
281 the population level.
282
283 *Statistical analyses*
284 Repeatabilities to test for within and among individual variation in heterophil-
285 lymphocyte (H:L) ratios, as well as lysis and agglutination scores were calculated according
286 to Lessells & Boag (1987). To stabilize variances and reach normal distributions, Box-Cox
287 transformations were performed for the response variables H:L ratio (lamda = 0) and number
288 of feather lice (lambda = 0.25; Box and Cox 1964).
289 Our aim was to assess the relationship between inbreeding and immunocompetence
290 estimates. However, first we carried out an analysis to correct for environmental effects that
291 may also influence our immune and ectoparasite estimates, and could hence lead to
292 differences between islands. To this end, we performed individual-based generalized linear
293 mixed model (GLMM) analyses including all sampled individuals, to account for any sex, year
294 or species effects. We included sex, year and species as fixed effect variables, species
295 nested within island as random effect, and performed separate analyses for each of the four
296 response variables, i.e. agglutination, lysis, H:L ratio and number of feather lice, to obtain the
297 least squares means for these variables. We then used these least squares means to
298 investigate the relationship between levels of inbreeding and immunocompetence. We
299 carried out population-level generalized linear models (GLM) analyses using temporal Fst
300 and population-specific Fst as fixed effect variables and the least squares means from the
301 individual-based analyses as response variables, again performing a separate analysis for
302 each of the four responses. Qualitatively, using the least squares means instead of the true
303 population means for the four response variables did not provide any differing results (data
304 not shown). Therefore, for ease of interpretation, we used the true population means for
305 agglutination, lysis, H:L ratio and number of feather lice in our final models. We also

performed analyses including mean population He as a further explanatory variable, but omitted it in our final analyses because He and population-specific Fst were highly correlated ($r^2 = 0.96$, $p < 0.0001$) and He did not add any additional information to our model.

Not all information was available for all individuals. Sample sizes therefore varied slightly among analyses (Table 1 & 2). All analyses were performed using the software JMP Version 8 and SAS Version 9.1 (SAS Institute Inc., Cary, NC) with significance set to $\alpha = 0.05$.

RESULTS

White blood cell counts

Within-smear repeatability was 82.5% ($F_{19,20} = 12.9$, $p < 0.0001$) and between-smear repeatability 84.7% ($F_{21,22} = 16.4$, $p < 0.0001$), demonstrating that our white blood cell counts were reliable. Mean heterophil:lymphocyte (H:L) ratio varied considerably among islands with the two *M. macdonaldi* populations and the birds from Rábida showing the highest values (Table 1 & Fig. 2a).

Relative numbers of white blood cells (WBCs/RBCs) in blood smears were unrelated to the H:L ratio of individual smears ($r^2 = 0.03$, $p = 0.15$) and also showed no significant correlation with any of the genetic diversity (NA, AR, He) or inbreeding (Fst) measures (all p-values > 0.46 and r^2-values < 0.11, data not shown). Therefore, we did not proceed to determine the WBC/RBC ratio for the remaining samples (i.e. the entire data set).

Hemolysis-hemagglutination assay

Repeat tests of the 16 individuals that were analysed twice (but on different assay plates) showed that repeatability was 8% for the hemagglutination reaction ($F_{15,16} = 1.24$, $p =$

331 0.33), whereas it was 58% for the hemolysis reaction ($F_{15,15} = 4.57$, $p = 0.003$). Hence, most
332 of the variation in agglutination occurred within individuals.
333 Agglutination scores of individuals ranged from 3.5 to 11 (the maximum possible) with
334 an overall mean score of 8.8 (± SD 1.2) and lysis ranged from 0 to 7 with a mean of 3.4 (±
335 SD 1.2). Overall, lysis and agglutination scores varied among populations (Fig. 2b & 2c), with
336 a difference of nearly two scores in mean agglutination titres and 1.5 scores in mean lysis
337 titres (Table 1). Interestingly, no lysis occurred in any of the samples collected on Rábida.
338
339 *Ectoparasites*
340 We dust-ruffled 196 individuals from all 13 populations (Table 1) and collected a total
341 of 1,482 *Myrsidea* and 133 *Brueelia* individuals. *Myrsidea* occurred on all islands whereas
342 *Brueelia* lice were only found in samples from Española, Gardner-by-Española, Gardner-by-
343 Floreana, Marchena, Rábida and St. Fé (Fig. 2d). *Myrsidea* showed a prevalence of 95.4%
344 and the mean number of *Myrsidea* lice in the plumage of a mockingbird was 7.6 (SD 6.5;
345 range 0 – 35). On the six islands where *Brueelia* occurred, mean infestation of *Brueelia* was
346 1.26 (SD 1.7; range 0 – 10), and prevalence was 41%. Because parasite abundance has
347 been shown to correlate positively with island size in Darwin's finches (Lindstrom et al. 2004),
348 we also tested for an effect of island size on mean feather louse load in the different
349 populations but found no significant correlation ($r^2 = 0.21$, $p = 0.12$).
350
351 *Genotyping and genetic diversity estimates*
352 We genotyped blood samples from a total of 400 individuals (Table 1). Successful
353 genotypes were obtained for all individuals and loci except for one individual, which could not
354 be genotyped at one single locus. All 26 loci were in Hardy-Weinberg equilibrium in all
355 populations except in four cases: MpAAT96 and Nes03 in Gardner-by-Floreana, Nes04 in
356 Santiago and Nes22 in Marchena which showed an excess of homozygotes. Genotypic
357 disequilibrium was only detected for two locus pairs in one single population (MpAAT96 x

358 Nes03 and Nes05 x Nes01 in Gardner-by-Floreana). The mean number of alleles per locus
359 was 9.5 and ranged from 3 to 18 alleles. The mean number of alleles per population across
360 all 26 loci was 91 (range: 32-154, Table 1), mean allelic richness was 3 (range: 1.18-4.69,
361 Table 1) and mean He 0.4 (range: 0.06-0.65, Table 1).

362 Population-specific Fst values were very variable and high in some populations,
363 ranging from 0.1 for the least inbred population on St. Cruz to 0.9 for the most inbred
364 population on Champion (Table 1). As expected, larger islands/populations showed lower Fst
365 values than smaller islands ($r^2 = 0.68$, $p < 0.001$; Fig. 3). Population-specific Fst correlated
366 significantly ($r^2 = 0.52$, $p = 0.006$) with temporal Fst. However, as the two Fst values
367 represent two different measures of inbreeding, i.e. long-term versus short-term inbreeding,
368 they were both kept in the main analyses.

369

370 *Inbreeding, immunocompetence and ectoparasites*

371 The four different response variables did not correlate (all p-values > 0.12 and r^2-
372 values < 0.23), except for lysis and number of feather lice which correlated positively at the
373 population level ($r^2 = 0.36$, $p = 0.023$), but not at the individual level ($r^2 = 0.001$, $p = 0.78$). On
374 the four islands that were sampled in all three years to account for year effects, variation
375 between years in the four response variables did not differ significantly (all p-values > 0.07).

376 We found no significant effect of population-specific or temporal Fst on agglutination
377 or lysis score, H:L ratio or number of feather lice (Table 2 & Fig. 2a-d). Hence, contrary to our
378 prediction, we detected no evidence for either inbreeding estimates to affect any of our
379 immunocompetence estimates or ectoparasite load. These results did not change when we
380 only included the blood-sucking *Myrsidea* lice in our model, and hence omitted *Brueelia* (p-
381 values > 0.47), or added He as an additional fixed effect (all p-values for He > 0.22, data not
382 shown). However, the statistical power of the analyses was low, as reflected by the large
383 confidence intervals of the parameter estimates (Table 2; see Hoenig and Heisey 2001 for
384 interpretation of confidence intervals in the context of statistical power).

DISCUSSION

Inbreeding, immunocompetence and ectoparasites

Several studies support a link between inbreeding or reduced genetic diversity and susceptibility to pathogens (e.g. Coltman et al. 1999, Acevedo-Whitehouse et al. 2003, MacDougall-Shackleton et al. 2005, Cassinello et al. 2001) or immunocompetence (e.g. Whiteman et al. 2006, Reid et al. 2003, Hawley et al. 2005). Inbreeding may affect the immune system at many different levels, such as through deleterious mutations (that could lead to non-functional or less efficient proteins or cells) or a loss of heterozygous advantage, or by decreasing immune protein variation. Because decreased lymphocyte numbers (i.e. increased H:L ratio) may signal immunosuppression and because immune-compromised birds may be more affected by parasites, we predicted a positive relationship between levels of inbreeding and H:L ratio as well as feather louse load. We predicted a negative relationship with agglutination and lysis titres as more inbred birds may display less variable antibodies or deleterious mutations could affect the complement enzyme cascade. However, we detected no significant relationships between the different immune and ectoparasite estimates and either of our two inbreeding estimates (Fig. 2 & Table 2). The lack of such correlations is not uncommon, and there are several possible explanations for it.

The innate immune traits and ectoparasite load investigated here may indeed not be affected by inbreeding in the Galápagos mockingbirds. In a recent review on heterozygosity-fitness correlations in animal populations, 24% of the correlations were non-significant (Chapman et al. 2009), indicating that such results are common. Although positive heterozygosity-fitness correlations have been widely reported (for reviews see e.g. Crnokrak and Roff 1999, Keller and Waller 2002, Reed and Frankham 2003), some fitness traits, such as parasite resistance, are in many cases unrelated to genetic diversity (Poulin et al. 2000a,

411 Poulin et al. 2000b, Hedrick et al. 2001, Cote et al. 2005, Coltman and Slate 2003, Stevens
412 et al. 1997, Taylor and Jamieson 2007). Rather than being associated with inbreeding or
413 heterozygosity *per se*, genetic variation in immune traits or resistance to parasites could be
414 associated with specific alleles (Spielman et al. 2004) such as MHC (Eizaguirre et al. 2009,
415 Bonneaud et al. 2005). Thus, a candidate-gene approach might be more fruitful for future
416 research to detect possible gene-resistance correlations in mockingbirds.

417 Alternatively, if different measures of immunity are traded-off against each other,
418 possible correlations between inbreeding and e.g. adaptive immune response would have
419 gone undetected in this study. If inbreeding impairs e.g. specific antibody response, there
420 could be a shift away from adaptive immunity towards greater reliance on innate immunity,
421 possibly reflected by the high NAb and complement enzyme levels found here (also see
422 discussion below). H:L ratios did not seem to be affected by either of the two inbreeding
423 measures here (Table 2) even though WBC levels have been shown to be under genetic
424 control in other organisms (e.g. Mahaney et al. 2005, Sun et al. 2008). H:L ratios are altered
425 by inflammation or stress and their levels therefore also reflect an individual's history of
426 pathogen exposure. Such variation in H:L ratios may have obscured our ability to detect a
427 genetic relationship since we could not account for the individuals' history of infection with
428 this dataset.

429 Ectoparasites are only a subset of the parasites that infest a host, and as we did not
430 examine other parasites such as endoparasites which are more likely to respond to subtle
431 differences in the immune response (Wakelin 1996), we cannot rule out that inbreeding may
432 have an effect on other parasites or pathogens. However, a recent health survey did not
433 detect any blood parasites in *Mimus trifasciatus* (Deem et al. 2011).

434 The Galápagos Islands are exposed to strong environmental variation and pathogen
435 pressure is known to fluctuate in such changing environments (Salam et al. 2009, Moyer et
436 al. 2002). Therefore, mockingbirds might have adapted their innate immune systems to this
437 wide range of conditions and purged some of their genetic load associated with immune traits

438 (Crnokrak and Barrett 2002) resulting in the true absence of a relationship between
439 inbreeding and immunocompetence (e.g. Visscher et al. 2001, Wiehn et al. 2002). If purging
440 of recessive deleterious alleles obscured a potential heterozygosity-fitness relationship (Reed
441 and Frankham 2003, Wiehn et al. 2002), we may have expected that populations with
442 relatively recent, fast inbreeding and strong genetic drift, such as the two small, endangered
443 *M. trifasciatus* populations (Hoeck et al. 2010a), would show lower immunocompetence or
444 more ectoparasites than populations with a long history of low genetic diversity (such as the
445 populations on St. Fé or Española, Hoeck et al. 2010b). We did not detect any such pattern
446 for the immune components measured here (Table 2 & Fig. 2), suggesting that purging is not
447 a very likely explanation for our findings.

448 Alternatively, island systems, such as the Galápagos, are relatively pathogen-poor
449 environments (McCallum and Dobson 1995, Frankham 1997, Wikelski et al. 2004) where
450 pathogen pressure may not be sufficient for inbreeding to show a detectable effect on
451 immunocompetence or where such effects could only become relevant under stressful
452 environmental conditions (Arcese 2003, Nelson et al. 2002) when immune responses need to
453 be traded-off against other traits (e.g. Hasselquist et al. 1999, Nelson et al. 2002, Zuk and
454 Stoehr 2002). The fact that others have found evidence for a relationship between genetic
455 diversity and pathogen load in other Galápagos birds (Whiteman et al., 2006) makes this
456 explanation less likely. Also, lower pathogen pressure will not necessarily mask inbreeding
457 depression in measures of innate immunity.

458 Although our sample size was larger than in comparable studies in Galápagos which
459 did detect significant effects of genetic diversity or population size on immune parameters
460 (Whiteman et al. 2006, Lindstrom et al. 2004), it is possible that some of the variation in
461 ectoparasite infestation or immune estimates we observed is due to unaccounted
462 environmental variability (rainfall, temperature, food availability etc.), even though we
463 detected no significant differences in parameter estimates between years for the four islands
464 that were sampled repeatedly. However, if such environmental effects are large in

comparison to the heterozygosity-associated signal, then the signal will be difficult to detect statistically.

The large confidence intervals associated with our estimates (Table 2) make it clear that, while we have no evidence for an association between genetic diversity and immune function, we also have no evidence that in fact such a relationship is absent. Furthermore, severe parasite infestation or disease might lead to mortality or reduced mobility and, hence, seriously sick birds might have gone undetected in our study given our capture methods. Also, we would not have detected selection that occurs early in life because we did not sample any nestlings or fledglings.

Immunocompetence measures

We did not detect any correlation between NAb levels and complement-mediated lysis or leucocyte numbers, supporting the general idea that they serve as independent measures of immunocompetence (Mendes et al. 2006, Matson et al. 2005). However, the low repeatability detected for lysis and especially agglutination cautions against such a statement. Such a low repeatability is surprising as the hemolysis-hemagglutination test has previously been shown to be a highly repeatable assay (Matson et al. 2005). We do not know what caused the low repeatability in our study. Our finding highlights the need for an independent assessment of the repeatability of this method in each individual study, something that is oftentimes omitted or not discussed.

The mean levels of agglutination in the mockingbirds (Table 1) were higher or at the high end of recently reported values from a range of birds (Matson 2006, Parejo and Silva 2009, Matson et al. 2005, Mendes et al. 2006), suggesting that Galápagos mockingbirds have high levels of natural antibodies. NAb levels are comparable to the ones found in another Galápagos endemic, the Galápagos Hawk (Whiteman et al. 2006). However, while the hawks showed no lysis reactions, all but one mockingbird population showed lysis (Table 2). The high NAb and relatively high complement enzyme titres found here could indicate that

492 the mockingbirds are equipped with a strong first line of defence. A shift in the immune
493 defence strategy towards innate as opposed to acquired immune responses has previously
494 been suggested in a study that detected increased innate defences of insular birds in
495 comparison to their continental relatives (Matson 2006).
496
497 *Conclusions*
498 We investigated whether inbreeding at the population level affects
499 immunocompetence in Galápagos mockingbirds, including short- and long-term inbreeding
500 measured with recent and historic samples from populations of different sizes and inbreeding
501 levels and examining three different measures of innate immunocompetence. We found no
502 effects of short-term or long-term inbreeding on innate immunity or ectoparasite load.
503 Unfortunately, despite an extensive data set our statistical power was low. The relatively high
504 levels of agglutination and lysis detected here suggest that the mockingbirds may have a
505 strong first line of defence against invading pathogens. However, as measures of
506 agglutination and lysis showed very low repeatabilities, these conclusions remain tentative.
507 Based on the results from this study, we are unable to predict how the mockingbirds would
508 cope with new, introduced pathogens. This stresses the importance of future avian health
509 surveys and disease control in the Galápagos.
510 The two endangered Floreana mockingbird (*M. trifasciatus*) populations did not show
511 lower levels of natural antibodies, complement enzymes or higher heterophil-lymphocyte
512 ratios than the other mockingbird populations investigated here. This suggests that *M.*
513 *trifasciatus* does not suffer from immunosuppression in comparison to the more outbred and
514 more widely distributed mockingbird species. In a recent study on the Floreana mockingbird,
515 however, significant differences were found in different health parameters between the two
516 satellite populations, suggesting that the genetically more diverse population on Gardner
517 Island is clinically healthier than the smaller and more inbred Champion population (Deem et

518 al. 2011). Whether this results in lower fitness and survival in the Champion in comparison to
519 the Gardner population remains subject to further investigations.
520
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534

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FIGURE LEGENDS

Figure 1:

Distribution of the four different mockingbird species (*Mimus* spp.) in the Galápagos archipelago with circles indicating the sites of sample collection. On St. Cruz (*PtA* and *GP*) and San Cristóbal (*Sur* and *Nor*) samples were obtained from two different locations.

Figure 2:

The three estimates of immunocompetence and feather louse numbers shown as a function of population-specific Fst, a measure of inbreeding, with values of Fst increasing from left to right (real numbers are given in Table 1 and Figure 3). To improve visual representation, points that overlapped (on the x-axis) were slightly moved. Bars represent the standard errors of the means. a) The mean heterophil-lymphocyte (H:L) ratio of each population. b) Mean lysis and c) mean agglutination scores for each population, with values corrected for the chicken control sample. d) The mean number of *Myrsidea* (closed circles) and *Brueelia* (open circles) lice per population. *Brueelia* did not occur in all populations. The total mean number of feather lice per population is shown in Table 1.

Figure 3:

Population-specific Fst as a function of the natural logarithm of island size (in ha; actual values are given in Table 1) with island size decreasing from left to right. Points that overlapped on the x-axis were slightly moved to improve visual representation. The two Fst estimates for St. Cruz and San Cristóbal represent the two different sampling sites on these islands. Bars show the minimum and maximum estimates for Fst as calculated in 2mod (Ciofi et al. 1999).

TABLES

Table 1:

Populations studied with island size (log-scale, in hectares), estimates of genetic diversity and immunocompetence and the respective sample sizes (N, number of mockingbird samples). Measures of genetic diversity are based on 26 microsatellite loci: NA: number of alleles, AR: allelic richness, He: expected heterozygosity, pop. Fst: population-specific Fst as calculated in 2mod (Ciofi et al. 1999), temp. Fst: temporal Fst as calculated in Hoeck et al. (2010b). Mean lysis and agglutination scores are given (for chicken control corrected values see Fig. 2b & c), as well as mean heterophil-lymphocyte (H:L) ratio and mean number of total feather lice encountered per individual in each population.

		genetic diversity				inbreeding		hemolysis-hemagglutination			H:L ratio		# of feather lice	
Island	island size (ln; ha)	NA	AR	He	N	pop. Fst	temp. Fst	mean lysis score	mean agglutination score	N	mean	N	mean	N
Champion	2.25	32	1.18	0.06	48	0.90	0.28	3.27	9.33	24	0.20	62	6.83	12
Espanola	8.71	68	2.08	0.25	58	0.65	0.02	3.58	8.48	63	0.33	88	9.27	33
Gardner-by-Espanola	4.06	45	1.73	0.17	10	0.72	0.11	4.33	9.33	3	0.44	10	13.89	9
Gardner-by-Floreana	4.4	62	2.12	0.32	69	0.69	0.08	3.68	9.18	44	0.21	72	8.45	22
Isabela	13.04	128	3.77	0.48	32	0.27	0.02	2.95	9.19	16	0.21	29	8.07	14
Marchena	9.47	95	3.04	0.47	38	0.39	0.02	3.55	8.45	20	0.11	39	8.11	18
Rabida	6.21	96	3.29	0.51	21	0.32	0.05	.	9.13	15	0.32	21	12.50	10
SanCristobal-Sur	10.93	96	3.23	0.40	20	0.39	0.02	2.50	9.46	14	0.11	12	2.50	6
SanCristobal-Nor	10.93	79	2.78	0.37	17	0.47	0.02	3.42	9.31	13	0.14	17	6.67	15
Santiago	10.98	154	4.69	0.61	27	0.12	0.01	3.70	7.37	15	0.12	27	2.25	12
StCruz-Gar	11.5	133	4.37	0.63	22	0.14	0.01	3.17	8.35	17	0.23	38	8.75	16
StCruz-PtA	11.5	137	4.68	0.65	17	0.11	0.01	3.31	8.77	13	0.21	17	4.89	9
StFe	7.79	59	2.13	0.33	21	0.58	0.01	3.71	9.71	12	0.10	21	10.30	20
Total # of samples					400					269		453		196

852 **Table 2:**

853 GLMs for a) agglutination (n=13) and b) lysis scores (n=12), corrected for chicken control
854 sample, c) heterophil-lymphocyte (H:L) ratio (ln-transformed, n=13), and d) number of feather
855 lice (transformed to the power of 0.25, n=13).

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	Estimate	±SE	95%CI	t-value	d.f.	p-value
a) Agglutination						
Intercept	-1.57	0.56	-2.8 – -0.3	-2.82	1,10	
population-specific Fst	2.23	1.42	-0.9 – 5.4	1.56	1,10	0.15
temporal Fst	-2.24	4.76	-13.0 – 8.5	-0.47	1,10	0.65
b) Lysis						
Intercept	-0.91	0.35	-1.7 – -0.1	-2.64	1, 9	
population-specific Fst	1.11	0.89	-0.9 – 3.1	1.25	1, 9	0.24
temporal Fst	-1.21	2.97	-7.9 – 5.5	-0.41	1, 9	0.69
c) H:L ratio						
Intercept	-2.30	0.31	-2.9 – -1.6	-7.51	1,10	
population-specific Fst	0.47	0.79	-1.3 – 2.2	0.59	1,10	0.57
temporal Fst	0.88	2.63	-5.1 – 6.8	0.33	1,10	0.75
d) Feather lice						
Intercept	1.24	0.16	0.8 – 1.6	7.77	1,10	
population-specific Fst	0.59	0.41	-0.3 – 1.5	1.45	1,10	0.18
temporal Fst	-0.75	1.37	-3.9 – 2.4	-0.55	1,10	0.60

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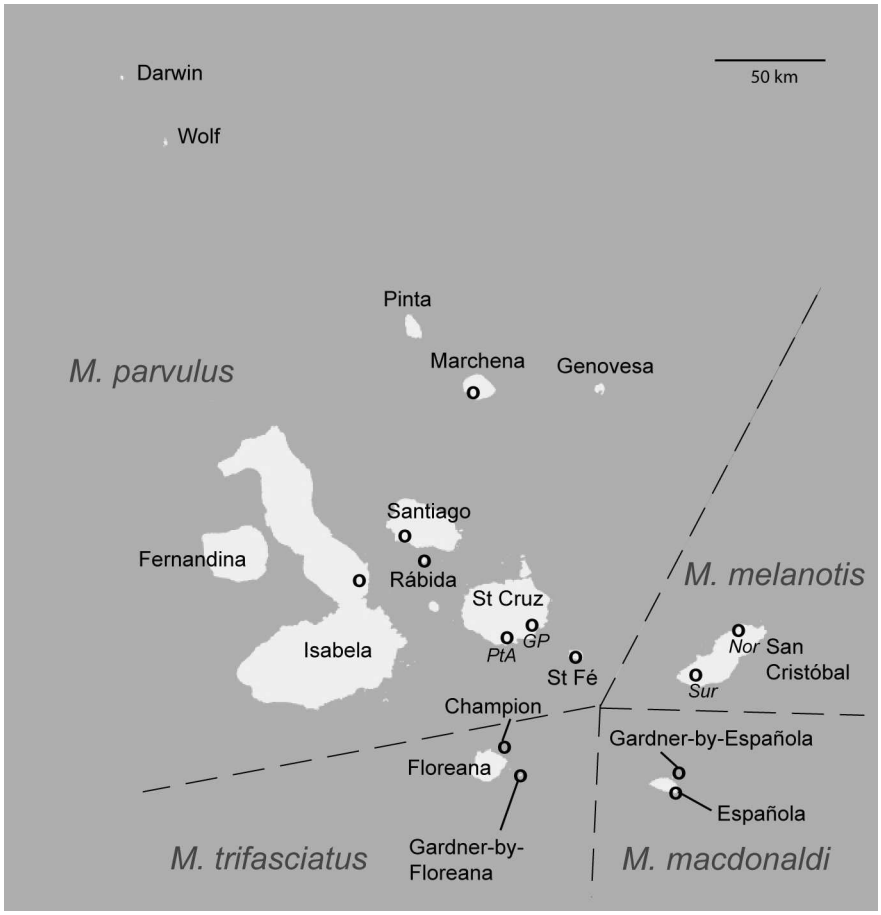
Figure 1:


Darwin
Wolf
50 km
Pinta
M. parvulus
Marchena
Genovesa
Santiago
Fernandina
Rábida
M. melanotis
St Cruz
GP
PtA
Isabela
St Fé
Nor
San
Cristóbal
Sur
Champion
Gardner-by-Española
Floreana
Española
M. trifasciatus
Gardner-by-
Floreana
M. macdonaldi

865 **Figure 2:**
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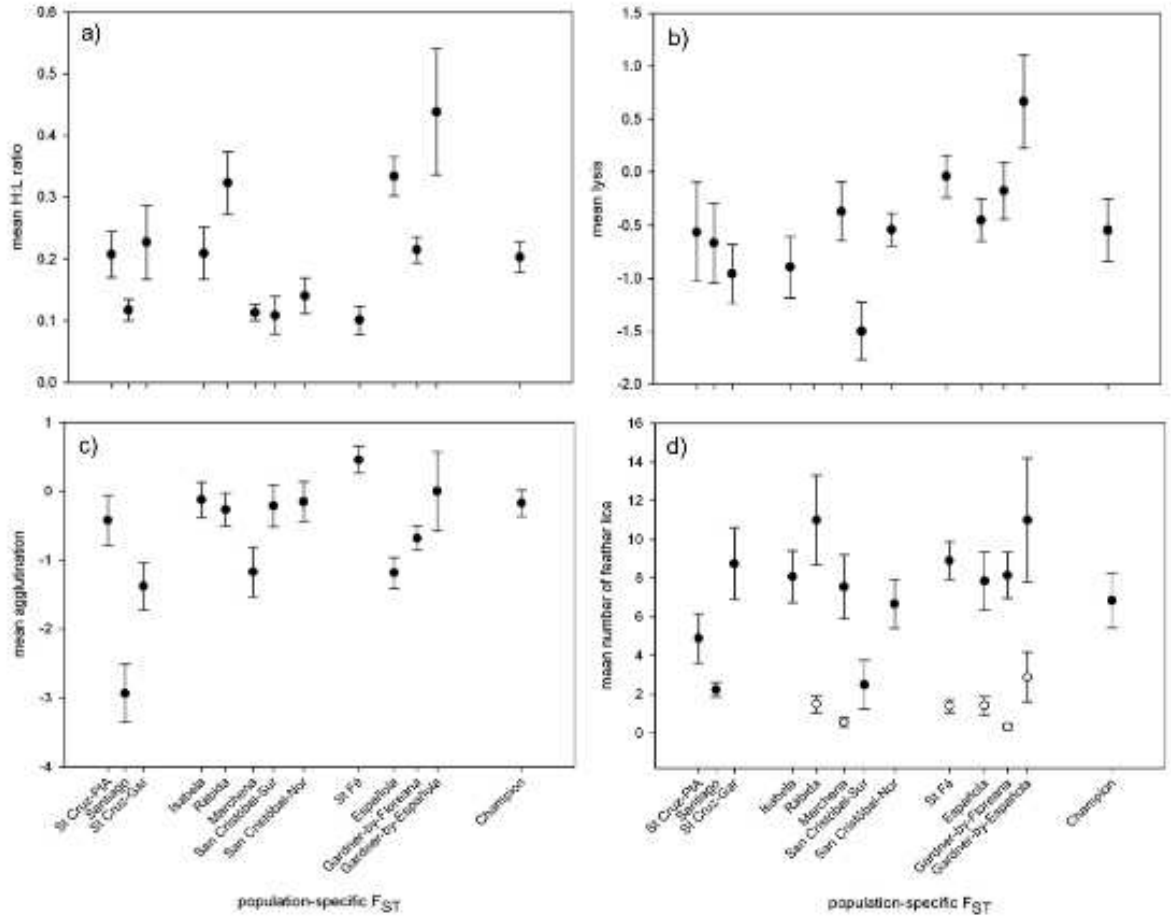

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870 **Figure 3:**
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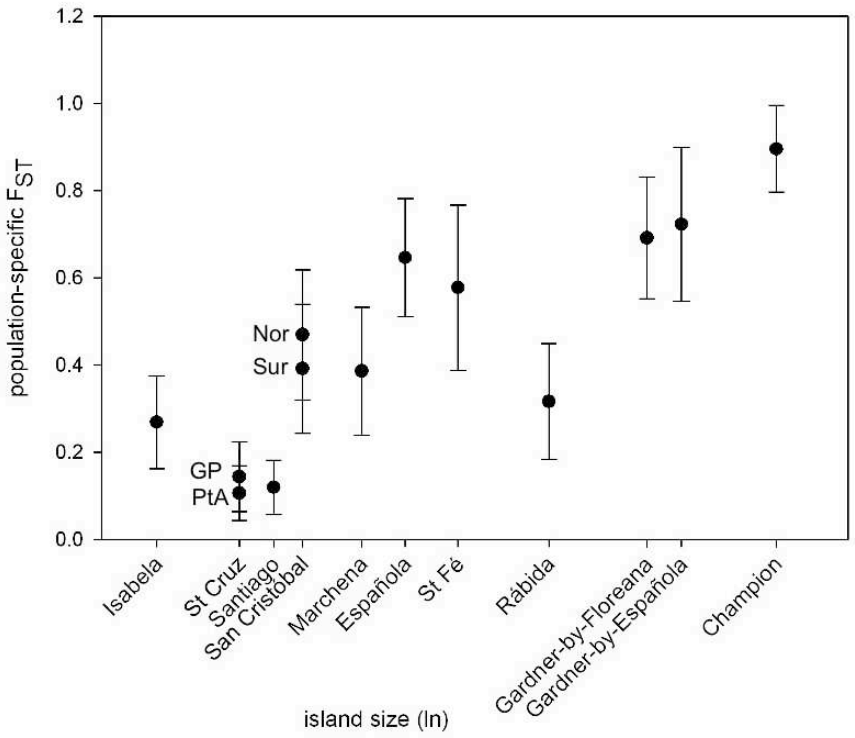

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